Isolation of Vitamin B_{12} Transport Mutants of *Escherichia coli*

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Escherichia coli KBT001, a methionine-vitamin B_{12} auxotroph, was found to require a minimum of 20 molecules of vitamin B_{12} (CN-B_{12}) per cell for aerobic growth in the absence of methionine. After mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine and penicillin selection, two kinds of B_{12} transport mutant were isolated from this strain. Mutants of class I, such as KBT069, were defective in the initial rapid binding of CN-B_{12} to the cell and were unable to grow in the absence of methionine even with CN-B_{12} concentrations as high as 100 ng/ml. The class II mutants possessed intact initial phases of CN-B_{12} uptake but were defective in the secondary energy-dependent phase. These strains were also unable to convert the CN-B_{12} taken up into other cobalamins. In the absence of methionine, some of these strains (e.g., KBT103) were able to grow on media containing 1 ng of CN-B_{12}/ml, whereas others (e.g., KBT041) were unable to grow with any of the CN-B_{12} concentrations used. Osmotic shock treatment did not affect the initial rate of uptake of CN-B_{12} but gave a substantial decrease in the secondary rate. Trace amounts of B_{12}-binding macromolecules were released from the cells by the osmotic shock, but only from strains such as KBT001 and KBT041 which possessed an active initial phase of CN-B_{12} uptake. These results are interpreted as being consistent with the view that the initial CN-B_{12} binding site which functions in this transport system is probably bound to the cell membrane.

The basic properties of the transport of cyanocobalamin, vitamin B_{12} (CN-B_{12}), in *Escherichia coli* K-12λ were described in the preceding paper (4). This process was shown to consist of an initial rapid phase of B_{12} uptake, which was essentially independent of the cell's energy metabolism, followed by a slower, energy-dependent, secondary phase. Mutants which were defective in one or other of these phases of B_{12} transport have been isolated from a methionine-B_{12} auxotroph of *E. coli*, and are described in this paper.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12λ was maintained and grown as described previously (4). The parent strain, KBT001, from which the B_{12} transport mutants were obtained was an *E. coli* K strain, of genotype F⁻ *pro lysA trp purE leu metE*, and was obtained from P. Cooper. Its methionine requirement could be satisfied by either methionine or vitamin B_{12}.

Bacterial growth. All of the bacterial strains were maintained on nutrient agar but were grown experimentally on the minimal medium of Davis and Mingioli (2), which was supplemented with 0.5% glucose, amino acids (100 μg/ml), and adenine (40 μg/ml) as required. When specified, vitamin B_{12} was usually

added at a concentration of 1 ng/ml. The cells were grown aerobically at 37 C, and growth was followed by measuring the optical density at 660 nm. The number of cells was counted with a Coulter Counter equipped with a 30-μm aperture tube. Suspensions containing 10^9 cells/ml gave optical densities at 660 nm of 0.8 and 0.73 for K-12λ and KBT001, respectively.

Cobalamin compounds. ⁶⁰Co-labeled CN-B_{12} (specific activity, ca. 1 mCi/μmole) was obtained from E. R. Squibb & Sons, New York, and ³H-labeled CN-B_{12} (specific activity, ca. 0.5 mCi/μmole) was obtained from Amersham/Searle. Pierrel, Milan, Italy, supplied the 5'-deoxyadenosyl cobalamin, (DBC) and methyl cobalamin (CH₃-B_{12}). CN-B_{12} and aquocobalamin (HO-B_{12}) were provided by Sigma Chemical Co. and Mann Research Laboratories, respectively.

Measurement of B_{12} uptake. The methods used have been described in detail previously (4) and, in general, consisted of incubating the cells with ⁶⁰Co-CN-B_{12} in 1% glucose-0.1 M potassium phosphate (*p*H 6.6), followed by separation of the cells from the reaction mixtures by filtration through membrane filters (0.45-μm pore size, Millipore Corp.). The filters were washed, dried, and counted in a liquid scintillation counter.

Formation of other cobalamins. In some experiments, the conversion of the added ⁶⁰Co-CN-B_{12} into other B_{12} compounds was determined. In these cases, after incubation with the labeled CN-B_{12}, the cells were har-

vested by centrifugation, suspended in a few milliliters of 10 mM potassium phosphate, (pH 6.8), containing 100 μg of each of unlabeled DBC, CH_3-B_{12}, CN-B_{12}, and HO-B_{12}, heated at 100 C for 5 min, and filtered through a membrane filter. The cobalamin compounds were extracted from the aqueous solution with phenol, reextracted into water, lyophilized, taken up in a small volume of water, and chromatographed on thin layers of silica gel. The solvent used was sec-butanol-water-25% ammonia (100:36:14, v/v; reference 6). After development, the regions of the silica gel containing the four separated cobalamin standards were transferred to scintillation vials, and their ^{60}Co content was counted. Corrections were made to compensate for the small amounts of ^{60}Co-CN-B_{12} which strayed into the chromatographic areas occupied by the other standard compounds.

RESULTS

Vitamin B_{12} requirements of E. coli KBT001.

Fifty-milliliter portions of the minimal medium, without methionine but containing 0.1 to 500 pg of vitamin B_{12} per ml (0.074 to 369 pM), were inoculated with about 5×10^8 methionine-depleted cells of E. coli KBT001. These cultures were grown aerobically at 37 C to their maximum densities, at which time the cell number was counted with a Coulter Counter. The results are shown in Figure 1. From the growth response to B_{12} between 20 and 100 pg/ml, it was calculated that the minimal B_{12} requirement for this strain, in the absence of methionine, was about 20 molecules per cell. In a preliminary experiment of this sort in which the cells were grown in the same medium, but in tubes without shaking, the minimal B_{12} requirement per cell was calculated to be about 11 molecules. Whether this represents a real difference in the B_{12} requirement between strongly aerobic and more microaerophilic growth is not known.

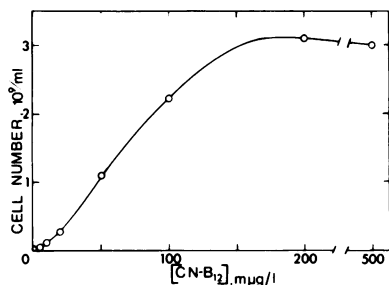### Mutagenesis and isolation of B_{12} transport

FIG. 1. Effects of CN-B_{12} concentration upon the growth of E. coli KBT001. Methionine-depleted cells were grown aerobically at 37 C on the minimal medium supplemented with various concentrations of CN-B_{12}. The number of cells per milliliter, at maximal cell density, is plotted against the initial CN-B_{12} concentration in the medium.

mutants by penicillin selection. The method used was that described by Adelberg et al. (1). The cells from a log-phase culture of KBT001 were harvested and incubated at 25 C for 15 min in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (minimal medium in which the phosphate salts were replaced with Tris-maleate) at pH 6.0 containing 100 μg of N-methyl-N'-nitro-N-nitrosoguanidine per ml. This treatment resulted in less than a 20% decrease in viability, and the survivors were allowed to segregate by growth overnight in nutrient broth. These cells were harvested, and washed with and resuspended in the minimal growth medium containing 0.5% glucose and all of the required growth factors, except that CN-B_{12} (0.2 or 1.2 ng/ml) was used instead of methionine. These cultures were incubated at 37 C until the cells entered the log phase and had undergone three doublings, giving a cell density of 1.4×10^8/ml. Penicillin G (1,000 units/ml) was added, and the incubation was continued for a further 2.5 hr until lysis was complete. The viable cell titer was 10^4 to 1.3×10^4/ml, and the cells were harvested by centrifugation and plated onto the minimal medium (solidified with 2% agar) containing all the essential nutrients including methionine, but no CN-B_{12}. The ability of 600 of the resulting colonies to utilize CN-B_{12} (0.6 ng/ml) in place of methionine was tested by replica plating. About 24% of these isolates were found to have lost the ability to grow on media in which CN-B_{12} was substituted for methionine.

In an attempt to identify strains with defective transport of vitamin B_{12}, the ability of these strains to grow upon media in which methionine was replaced with a high concentration of CN-B_{12} (25 ng/ml) was tested. Seven strains, KBT101 through KBT107, grew under these conditions, and these strains were found to be at least partially defective in the secondary, energy-dependent phase of B_{12} transport. The transport of vitamin B_{12} by a further 70 strains, KBT002 to KBT071, which were unable to grow on either high or low levels of CN-B_{12}, was examined directly. These strains were grown on the minimal medium with methionine and exposed to ^{60}Co-CN-B_{12} for 60 min, and the amount of ^{60}Co taken up by the cells was determined. Those strains which took up appreciably less ^{60}Co-CN-B_{12} than the parent KBT001 were selected for further study.

General properties of the B_{12}-transport mutants.

The conversion of ^{60}Co-CN-B_{12} into other cobalamins was examined in all of the strains which were unable to grow on low concentrations of CN-B_{12}. The cells were grown on the minimal medium containing methionine (100 μg/ml) and

TABLE 1. *Properties of some of the mutant strains which were isolated from E. coli KBT001*

Strain no.	B_{12} transport mutant class	Growth with $CN-B_{12}$ in absence of methionine	Activity of B_{12} transport		Per cent of $^{60}Co-CN-B_{12}$ taken up by the cells which co-chromatographed as[a]			
			Initial phase	Secondary phase	$CN-B_{12}$	DBC	$HO-B_{12}$	CH_3-B_{12}
KBT001	Parent	+	+	+	13	35	39	13
KBT020	I	−	−	ND[b]	18	33	42	7
KBT026	I	−	−	ND	26	32	29	13
KBT069	I	−	−	ND	25	48	19	8
KBT041	II	−	+	−	93	6	0	1
KBT101	II	±[c]	+	−	89	8	0	2
KBT103	II	±[c]	+	−	90	6	3	1
KBT065	None	−	+	+	6	34	53	6
KBT067	None	−	+	+	11	18	59	11
KBT039	None	−	+	+	6	27	43	24
KBT047	None	−	+	+	10	29	35	26

[a] Cultures were grown with 100 μg of methionine per ml and 3 nM $^{60}Co-CN-B_{12}$ in the medium. Abbreviations: $CN-B_{12}$, cyanocobalamin-vitamin B_{12}; $HO-B_{12}$, aquocobalamin; CH_3-B_{12}, methyl cobalamin; DBC, 5'-deoxyadenosylcobalamin.

[b] Not determined. It was not possible to measure the secondary uptake of $^{60}Co-CN-B_{12}$ in the absence of an initial phase.

[c] Growth in the absence of methionine only with $CN-B_{12}$ (> 10 ng/ml).

3 nM $^{60}Co-CN-B_{12}$. At maximal cell density, the cells were harvested, their cobalamin compounds were extracted and chromatographed, and the distribution of the ^{60}Co among $CN-B_{12}$, $HO-B_{12}$, DBC, and CH_3-B_{12} was measured. The properties of some representative strains are listed in Table 1. It was found that the B_{12} transport mutants fell into two distinct classes. The class I mutants had a defective initial phase of B_{12} uptake and took up essentially no vitamin B_{12}, whereas the class II mutants apparently lacked only the secondary energy-dependent phase of B_{12} uptake and seemed to possess an intact initial phase. Of particular interest was the observation that the class II mutants were essentially unable to convert the $^{60}Co-CN-B_{12}$ taken up into other cobalamins. Enough $^{60}Co-CN-B_{12}$ was taken up by the class I mutants during growth on methionine to show that the formation of other cobalamins was apparently normal in these strains. Also included in Table 1 are examples of other mutants which had intact B_{12} transport systems, but which showed unusual cobalamin contents, some high in CH_3-B_{12}, others high in $HO-B_{12}$. These strains were presumably defective in some other part of the methionine synthetase system, which may be reflected in these abnormal cobalamin distributions.

Growth and B_{12} uptake in strains KBT001, KBT041, KBT069, and KBT103. Strains KBT041, KBT069, and KBT103 were selected, as being representative of the two mutant classes, for further study. The growth responses of these strains and of their parent, KBT001, to either methionine (100 μg/ml) or $CN-B_{1}$· (0.1 to 100 ng/ml) are shown in Fig. 2 and 3. Before growth
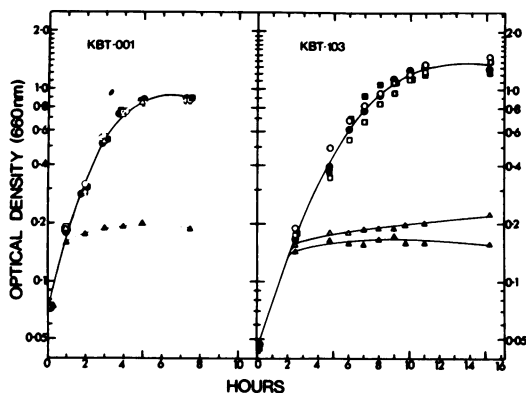

FIG. 2. *Time course of growth of E. coli strains KBT001 and KBT103 on the minimal medium containing either methionine or various $CN-B_{12}$ concentrations. Growth conditions: aerobic, 37 C, methionine-depleted cells. Additions: methionine, 100 μg/ml (O); $CN-B_{12}$, 100 ng/ml (●); 10 ng/ml (□); 1 ng/ml (■); 0.1 ng/ml (△); none (▲).*

with $CN-B_{12}$, the methionine available to the cells was depleted by growth on the minimal medium in the absence of both methionine and $CN-B_{12}$. The degree of methionine depletion of the cells seemed to be proportional to the length of the lag phase before growth occurred in media with $CN-B_{12}$ and no methionine. This was noticeable, obviously, only in those strains which were able to grow with $CN-B_{12}$, i.e., KBT001 and KBT103. The results in Fig. 2 show that the parent strain KBT001 was able to grow equally well on all concentrations of vitamin B_{12}, but not at all in the absence both of methionine and of
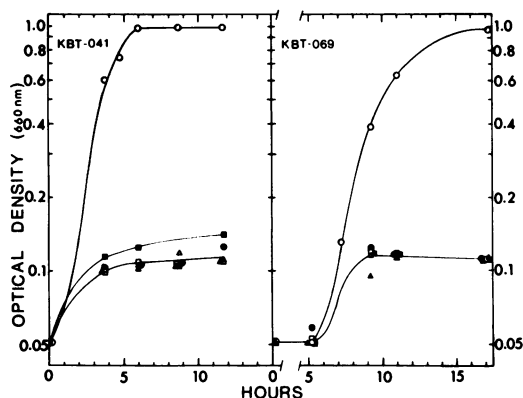
FIG. 3. *Time course of growth of E. coli strains KBT041 and KBT069 on the minimal medium containing either methionine or various CN-B_{12} concentrations. Growth conditions: aerobic, 37 C, methionine-depleted cells. Additions: methionine, 100 $\mu g/ml$, (O); CN-B_{12}, 100 ng/ml (●); 10 ng/ml (□); 1 ng/ml (■); 0.1 ng/ml (△); none (▲).*

vitamin B_{12}. Strain KBT103, a class II mutant, was unable to grow in the absence of methionine on media which contained only 0.1 ng of CN-B_{12}/ml, but grew well when the vitamin B_{12} concentration was increased to 1 ng/ml. Figure 3 shows that strains KBT041 and KBT069 were unable to utilize CN-B_{12}, at any of the concentrations tested, for growth on the minimal medium in the absence of methionine.

Typical time courses of ^{60}Co-CN-B_{12} uptake (at 3 nM ^{60}Co-CN-B_{12}) are compared in Figure 4. The change in scale for the parent strain, KBT001, should be noted. The class II mutants, KBT041 and KBT103, possessed the initial rapid phase of B_{12} uptake only, with essentially no additional uptake of vitamin B_{12} after 1 min. KBT069, a class I mutant, clearly lacked appreciable activity in either phase of B_{12} uptake.

Osmotic shock treatment of E. coli K-12λ. Figure 5 shows the effects of an osmotic shock [using the ethylenediaminetetraacetic acid Tris-hydrochloride (pH 8) procedure of Neu and Heppel (7)] upon the time course of ^{60}Co-CN-B_{12} uptake by cells of *E. coli* K-12λ. The rate of the secondary energy-dependent phase of uptake was greatly diminished by this treatment. Addition of a concentrated solution (obtained by lyophilization and solution in distilled water) of the compounds, released by the shock treatment back to the cells, resulted in some restoration of the rate of secondary B_{12} uptake. The initial rate of B_{12} uptake was apparently not affected by the shock treatment, and this was confirmed by measuring the initial rates of uptake directly in a similar experiment (Fig. 6).
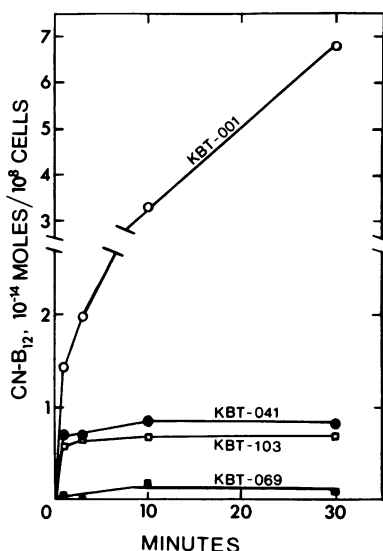

FIG. 4. *Time course of ^{60}Co-CN-B_{12} uptake by E. coli strains KBT001, KBT041, KBT103, and KBT069. Reaction conditions: 30 C, aerobic, 0.1 M potassium phosphate (pH 6.6), 1% glucose, 0.89 nM ^{60}Co-CN-B_{12}. Cells were preincubated for 15 min before addition of ^{60}Co-B_{12}. Samples (5 ml) were taken. Each reaction mixture contained 10^8 to 1.5×10^8 cells/ml. Strains: KBT001, O; KBT041, ●; KBT103, □; KBT069, ■.*
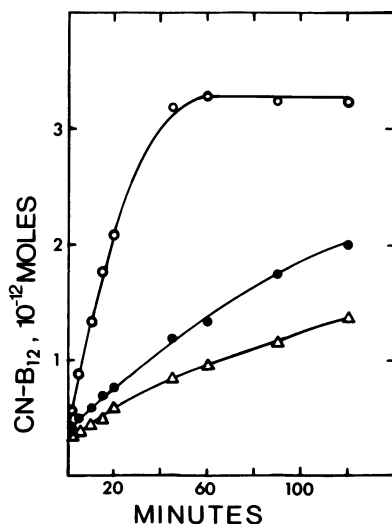

FIG. 5. *Effects of osmotic shock treatment on the uptake of ^{60}Co-CN-B_{12} by cells of E. coli K-12λ. Incubation conditions: aerobic, 37 C, 0.1 M potassium phosphate (pH 6.6), 1% glucose, 5.1 nM ^{60}Co-CN-B_{12}. Preincubation was 30 min. Reaction mixtures were 20 ml; samples were 1 ml. Whole cells (final OD_{660}, 0.60), O; shocked cells (final OD_{660}, 0.55), △; shocked cells (final OD_{660}, 0.55) plus "shocked factor," ca. 100 μg of protein contained in a concentrated solution obtained*
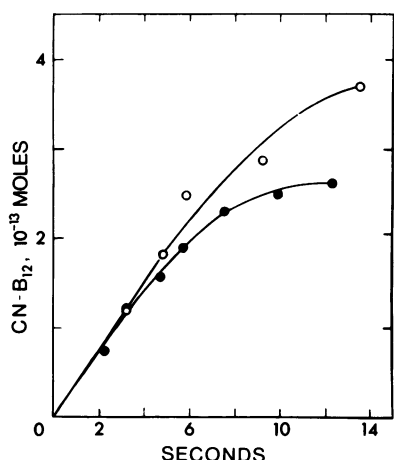
FIG. 6. *Effects of an osmotic shock treatment on the initial rate of ^{60}Co-CN-B_{12} uptake by cells of E. coli K-12λ. Reaction conditions: aerobic, 25 C, 0.05 M potassium phosphate (pH 7), 1% glucose, 0.4% Hyflo Super Cel, 1.47 nM ^{60}Co-CN-B_{12}, and cells to an OD_{660} of ca. 0.2. Reaction mixtures were 50 ml; samples were 2.5 ml. Preincubation was for 15 to 30 min. Care was taken to use the same number of cells in each reaction mixture. Whole cells, ○; shocked cells, ●. The flow assay method was used (4).*

By means of equilibrium dialysis experiments, it was found that among the compounds released from the cells by the osmotic shock was a macromolecular component which bound ^{60}Co-CN-B_{12}. This prompted a search for the release of B_{12} binding macromolecules upon osmotic shock treatment of the B_{12} transport mutants. One-liter cultures were grown on the minimal medium at 37 C; after harvest, the cells were subjected to osmotic shock treatment. The shocked cells were removed by centrifugation, and the supernatant solutions were lyophilized, dissolved in a few milliliters of distilled water, and dialyzed overnight at 2 C against two changes of 2 liters of 10 mM potassium phosphate (pH 6.8). B_{12} binding components present in these solutions were assayed by experiments of the Hummel-Dreyer type (5). Samples of these solutions containing 0.65 to 3.2 mg of protein, 4 nM ^{3}H-CN-B_{12}, and 10 mM potassium phosphate (pH 6.8) were applied to columns (1 by 35 cm) of Sephadex G-25 which had been equilibrated with 10 mM potassium phosphate (pH 6.8) containing 4 nM ^{3}H-CN-B_{12}. The columns were eluted with the same buffer ^{3}H-B_{12}

from the supernatant solution after the shock treatment, ●. The "shocked factor" and the shocked cells were incubated together at 37 C in a very small volume of solution before being transferred to the larger volume preincubation mixture.

solution, and portions were assayed for tritium and for absorbance at 280 nm. The results (Fig. 7) indicate that the osmotic shock treatment released some B_{12} binding material from those strains, KBT001 and KBT041, which possessed the initial phase of B_{12} uptake. No significant B_{12} binding activity was apparently released from strain KBT069 which was defective in the initial B_{12} transport process.

DISCUSSION

E. coli KBT001, a methionine-B_{12} auxotroph, required a minimum of 20 molecules of vitamin B_{12} per cell for growth on a minimal medium without methionine. If the kinetic characteristics of the B_{12} transport system in this strain are essentially the same as shown for *E. coli* K-12λ in the preceding paper (reference 4; K_m, 5 nM; V_{max}, 56 molecules per sec per cell), it can be concluded that the growth rate of KBT001, in the absence of methionine, should not be limited by the initial rate of B_{12} uptake at B_{12} concentrations in the medium greater than 1 pg/ml (0.74 pM). In other words, below 0.74 pM CN-B_{12}, the cell's generation time would be determined by the time required to take up 20 B_{12} molecules per cell. Thus, it was not surprising that, in the ab-
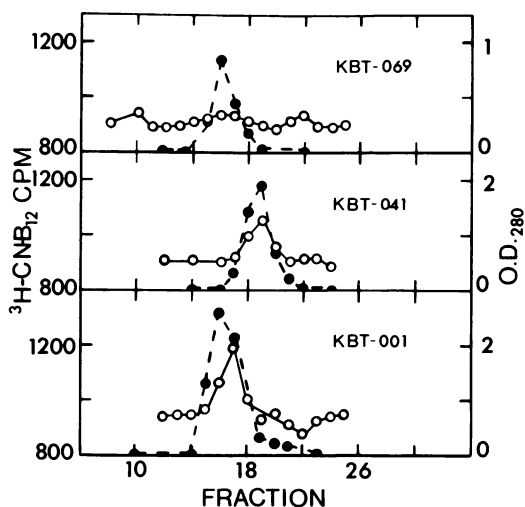

FIG. 7. *Assay for ^{3}H-CN-B_{12} binding activity released from cells of E. coli KBT001, KBT041, and KBT069, by osmotic shock treatment. Macromolecular concentrates of the material released from the cells were taken up in 10 mM potassium phosphate (pH 6.8)-4 nM ^{3}H-CN-B_{12} and were chromatographed on columns of Sephadex G-25 which were equilibrated and then eluted with 10 mM potassium phosphate-4 nM ^{3}H-CN-B_{12}. Fractions (1 ml) were collected. Symbols: OD_{280} (●); counts per minute of ^{3}H-CN-B_{12} (○). The amounts of protein applied to each column were: KBT001, 3.2 mg; KBT041, 1.7 mg; KBT069, 0.65 mg.*

sence of methionine, the growth rates of KBT001, in the minimal medium containing CN-B_{12} concentrations which ranged from 100 pg to 100 ng/ml, were the same. When cells of this strain, which had been grown on methionine, were subjected to methionine starvation before their transfer to a medium containing CN-B_{12}, there was often an appreciable lag phase before growth occurred in the B_{12}-containing medium. This may well be a diauxic phenomenon and is similar to some of the observations of Dickerman et al. (3) with another methionine-B_{12} auxotroph.

After mutagenesis of KBT001 with nitrosoguanidine and penicillin selection of strains which were unable to utilize CN-B_{12}, a number of mutants were obtained with B_{12} transport systems which were apparently no longer adequate to meet the modest demands of 20 B_{12} molecules per cell. Some of these strains, including KBT041 and KBT069, were unable to grow on media without methionine and containing up to 100 ng B_{12}/ml. Other strains, such as KBT103, grew as well as the parent KBT001 on B_{12} concentrations above 1 ng/ml, but growth was essentially absent on 0.1 ng/ml.

The time course of uptake of ^{60}Co-CN-B_{12} by cells of KBT001 was found to be essentially the same as that described for K-12λ (4) and consists of an initial rapid phase of uptake followed by a slower secondary phase. The B_{12} transport mutants which were isolated were found to be defective in either one or the other of these phases.

The class I B_{12} transport mutants were defective in the initial phase of uptake. This phase was essentially independent of the cellular energy metabolism (4), and we believe that it consists of B_{12} binding to sites close to the cell surface, which are the primary B_{12} binding sites involved in B_{12} transport. The cellular location of these sites is of importance in understanding the transport of vitamin B_{12}. Osmotic shock treatment, which is usually used to release proteins from the periplasmic space, of the various strains released some B_{12} binding macromolecules in some cases. The evidence that these macromolecules are involved in B_{12} transport is that detectable B_{12} binding activity was released only from those strains which possessed an intact initial phase of B_{12} uptake, i.e., from strains KBT041 and KBT001, but not from KBT069. However, the amount of B_{12} binding material released was very small. Assuming that this binding component had the same kinetic characteristics as the sites involved in the initial uptake of vitamin B_{12}, and that it had not been inactivated by our procedures, we calculated that not more than one B_{12} binding site per cell was released from

KBT001 by the osmotic shock. Accordingly, we believe that this small release of B_{12} binding sites represents the dissociation of a small fraction of binding sites which are usually fairly firmly bound to the cell surface, rather than the more quantitative release which might be expected of a component which normally inhibits the periplasmic space. Consistent with the view that only a small proportion of the B_{12} binding sites were released was the observation that the initial rate of B_{12} uptake was essentially not affected by the osmotic shock. Some recent experiments, as yet unpublished, which indicate that the initial B_{12} binding sites are membrane bound, have shown that isolated cell membrane vesicles from strains K-12λ, KBT001, KBT041, and KBT103, which possess intact initial phases of B_{12} transport, were able to take up ^{60}Co-CN-B_{12}. Membrane vesicles from strains, such as KBT069, which lacked the initial phase of transport, were unable to take up labeled CN-B_{12}.

The class II B_{12} transport mutants were defective in the secondary phase of B_{12} uptake but had intact initial uptake processes. The secondary phase of B_{12} transport in *E. coli* has been shown to be coupled to the energy metabolism of the cell. It was of interest to find that these class II mutants were unable to convert the ^{60}Co-CN-B_{12} taken up into other cobalamins. This might indicate that the energy coupling system in B_{12} transport consists of the conversion of the CN-B_{12} into other cobalamins, such as the coenzyme, DBC. In the preceding paper (4), however, evidence was presented that there was no such obligate coupling between transport and chemical conversion. Another possibility is that the secondary uptake consists of an energy-dependent release of the B_{12} into the interior of the cell from a membrane-bound site. In this case, the B_{12} taken up by class II mutants would remain membrane bound and would not be available to the soluble enzymes involved in DBC formation. These possibilities are currently being investigated. The nature of the energy-donor system in B_{12} transport is at present unknown. Both glucose and D-lactate stimulated the rate of the secondary phase of B_{12} uptake in whole cells, but neither of these compounds, nor phosphoenolpyruvate, gave any marked stimulation of B_{12} uptake by membrane vesicles (*unpublished data*). The osmotic shock treatment greatly diminished the rate of the secondary phase of B_{12} transport and, whereas this may have been due to the loss of a coupling protein, it was probably the result of the loss from the cells of small molecules, such as nucleotides, which are required for energy metabolism.

The lack of mutants with defective initial

phases of B$_{12}$ uptake and possessing demonstrable secondary transport argues in favor of these two phases being sequential parts of a single B$_{12}$ transport system, rather than two independent processes.

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